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Molecular identification of edible insects of Nagaland

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Abstract
Entomophagy practices should rely on proper identification of insects, which are usually classified relying on morphological keys and traditional knowledge practices. This may lead to misidentification of edible insects. Hence, the present study, DNA barcoding was used to identify and documentation of edible insects. In the primary study 7 edible insects were collected and identify by morphological and confirmed by DNA barcoding method and the species level confirm through phylogenic analysis using BLAST. COI gene sequence was submitted to Gene Bank under the accession Number *Choroedocus illustris* (MN82982), *Chondracris rosea* (MN829822), *Samia ricini* (MN829823), *Tagasta indica* (MN829824)*: Tessaratoma papillosa* (MT009020), *Batocera rubus larva* (MT089705) and *Odontotermes longignathus* (MT009019).

Keywords: COI gene sequences, DNA barcode, edible insects

Introduction
Insect as food is common among the ethnic people of Northeast India mainly among tribes of Arunachal Pradesh, Assam, Manipur and Nagaland. Studies have revealed that almost 255 insect species are used as food by different tribes of India. Documentation of edible insect in various state of India have shown that Arunachal Pradesh (158 Species of edible insect), Manipur (41 species of edible insects), Assam (38 Species of edible insects) and Nagaland (42 Species of edible insects) Meghalay (16 species) Kerala 5 species [1, 2]. The native people inhabiting in the north eastern state of India consume edible insect species at their different development stages (Adults, larva stages, pupa Eggs).These people use their traditional knowledge to determine which species to be eaten and at what stage. The People of different tribes select the edible insects on the basis of their traditional idea, taste and also regional and seasonal availability [3]. They likewise have a tremendous customary information on the viable use of consumable insects which are obtained through experience and normally passed on by oral conventions as a monitored mystery of specific families [4]**.** In the studies, Molecular tools used for identification of edible insects. Over the recent three decades, Mitochondrial DNA has been broadly analyzed [5] and demonstrated to be a significant tool in species delimitation as it has biological properties making it appropriate as a marker for molecular biodiversity. Limited DNA sequencing of the mitochondrial gene, for example, Cytochrome C oxidase I (COI) and other molecular makers have been utilized to recognize and find new species. A few investigations have indicated that a 648bp fragment of COI can be utilized as a DNA barcode to identification and recognize individual species [6]. Fragments of COI has been appeared to give high goals to recognize obscure species, along these lines expanding scientific classification based on biodiversity gauges [7]. Nonetheless, DNA barcoding proved to be a versatile tool with a variety of application, for example, by facilitating the association between different developmental stages in insects [8]. The methods of identification of edible insects and practices of entomophagy may not be known to other people and may disappear with that group of tribal. In the present study, the aim was to collect information about edible insect in certain Naga tribes and then the identified edible insect was confirmed by Molecular methods of DNA barcoding techniques.

Material and Methods
Insects were collected from local market in Dimapur, Nagaland and preserved in 100 % ethanol. DNA was extracted from body tissues using CTAB or Kit based methods. Genes were amplified using PCR. Each PCR reaction for testing the amplification efficiency and development of multiplex PCR assays for DNA barcode primers contained 1µl DNA template (25 ng), 2µl 10X reaction buffer, 0.5µl MgCl2 (50pM), 1µl dNTPs mix (10mM), 1µl forward primer (10pM), 1µl reverse primer (10pM), 0.5µl Taq polymerase (5 U/pi) and the final volume 25µl will be adjusted with molecular grade water. Primers are standard primers available for COI gene amplification.
COIF- GGTCAACAAATCATAAAGATATTGG- Tm 510C
COIR- TAAACTTCAGGGTGACCAAAAAATCA- Tm 530C

Phylogenetic Tree Analysis
A Phylogenetic tree was drawn based on distance neighbor tree-joining method and Maximum Likelihood method in Geneious v.9.0.2 [9] and also used BLAST analysis tool from NCBI. FAST format of the COI gene sequence were submitted in Gene Bank to get accession number from NCBI (https://www.ncbi.nlm.nih.gov/). The analysis of Sequence composition was performed with help of available tools in BOLD. Barcode sequences of the sampled specimen are available online in the dataset of MMLT20 present in

the Database of BOLD. (http://www.boldsystems.org).

Result

Entomophagy is an age old practice that continues to this day in many tribe's people in Nagaland, A total seven edible insects belonging to 7 families of 4 orders (Orthoptera, Lepidoptera, Coleoptera and Hemiptera) were collected from Dimapur, Nagaland. DNA extracted from tissue sample of 7 edible species. Most of amplified sequences were up to 450 to 680 bp length. BLAST was used to check homology of between the retrieved sequences in Gene Bank library. This help to identify sequence similarity across genomes. Analysis of homology using BLAST revealed that Sample-2 were 98.45% similar with voucher insects of *Choroedocus illustris* (KY83924.1) (Table-1, Fig-1) and Sample-3 have 98.67% match with the voucher specimens of *Chondracris rosea* (MK007252.1) (Table-2, Fig-2). Sample 4 COI sequences were analyzed in BLAST to revealed 100% pair wise matched with voucher samples of *Samia ricini* (MN120775.1) in NCBI (Table-3, Fig-3). Another BLAST analysis disclosed that the observed COI sequence of sample -5 showed 95.98% homology with voucher specimen of *Tagasta indica* (NC045930.1) (Table - 4, Fig-4). It indicates that observed sample 5 is *Tagasta indica*. In the table,-6, BLAST analysis showed that the observed sequence of sample 6 has 92.54 % homology with voucher specimen sequences in Gene bank from India. It reveals that the observed sample is Odontotermes longignathus (Table-5, Fig-5). BLAST result disclosed that the observed sample COI sequence of Tessaratoma sp showed 80.58 homology with voucher specimen of Tessaratoma papillosa (NC037742.1). It indicates that the observed samples are Tessaratoma papillosa (Table-6, Fig-6). In table- 7, BLAST analysis showed that the observed sequence of samples 8 has 92.47 % similar with voucher specimen sequence of *Batocera rubus* (KT314213.1) in the gene bank. The nucleotide sequences of the COI for the 7 species of edible insects were submitted to Gen Bank and subjected to a homology search using BLAST. A Phylogenetic tree based on COI sequence of the edible insects was constructed using neighbor tree joining and maximum likelihood methods. All edible insects COI sequence was submitted in NCBI under the accession number provided in table-8. Phylogenic of NJ analysis also depicted three species closely related *Choroedocus illustris, Chondracris rosea and Tagasta indica*. In the clad A, *Chondracris rosea* is resoled as a sister group to *Choroedocus illustris* and *Tagasta indica* as well as forms a monophyletic group (Fig-7). The pair wise distance was calculated by the MEGA X software. The inter specific nucleotide divergence between the seven edible species range from 6.76% to 17.72 (Table-9). The highest distance of 17.72 was obtained between *Tagasta indica* and *Tessaratoma papillosa* the shortest distance of 7.47 was obtained between *Choroedocus illustris* and *Chondracris rosea*. Sequences composition analysis by using BOLD system the highest mean value has registered in "T" bas pair 33.27 and followed by 26.16 (Adenine), 22.92 (Guanine),17.63 (Cytosine) (Tabl-10).DNA barcode images depicted in table-11.

Discussion

In Nagaland 42 to 60 insect species have been documented as edible insects by morphological identification methods, belonging to different family and orders. In the present study, molecular identification of selected edible insects (Grasshoppers: *Choroedocus illustris, Chondracris rosea, Tagasta indica, Samia ricini (silk worm), Tessaratoma papillosa* (Sting Bugs*), Batocera rubus larva* (wood borers) *and Odontotermes longignathus* (wings termites) was done by DNA barcoding method. These results could be explained by the findings of Sanket Tembe *et al*., (2014) [10] who also done selected Sting Bugs species identified by DNA barcoding methods and states that DNA barcode method is very useful and quick method for the insects to be identified at the species level [10]. Similarly, Shama Paraveen *et al*., (2015) have also done molecular identification of different life stage (Egg, different instar stages and adults) of *Tessaratoma javanica* (Thun bug) by DNA barcoding method. The first report revealed 670 bp COI gene sequences from tissues samples of *Tagasta indica* in India. Three grasshopper have been done COI gene sequences screen from tissues samples [11]. The present findings supported that of Muhammedali *et al*. (2017) [12] who have done for 589 bp of COI gene sequence screened from Grasshopper *Conocephlus dorsalis* and received accession number KX503055 from Genebank [12]. Additional, Sanket tembe *et al*., (2014) [10] who have added databases of mitochondrial cytochrome C oxidase I (mtCOI) sequences from the forty three species of indigenous true bugs. COI gene sequence screen from larva stages of *Samia ricini* (Silk worm) [10] and *Batocera rubus* larva stages parallel result support from the observations of Ekrem *et al*., 2010, who have reported that DNA barcoding allows the inclusion of all life stages in biodiversity assessments (Ekrem etal. 2010) [13].

Table 1: BLAST analysis of *Choroedocus illustris* with voucher specimens in Gene bank

S. No	Specimen Name	Total Score	Query Cover	E value	Per identification	Accession
1	Choroedocus illustris	1067	87	0.0	98.45	KY83924.1
2	Choroedocus illustris	1056	86	0.0	98.44	KY837806

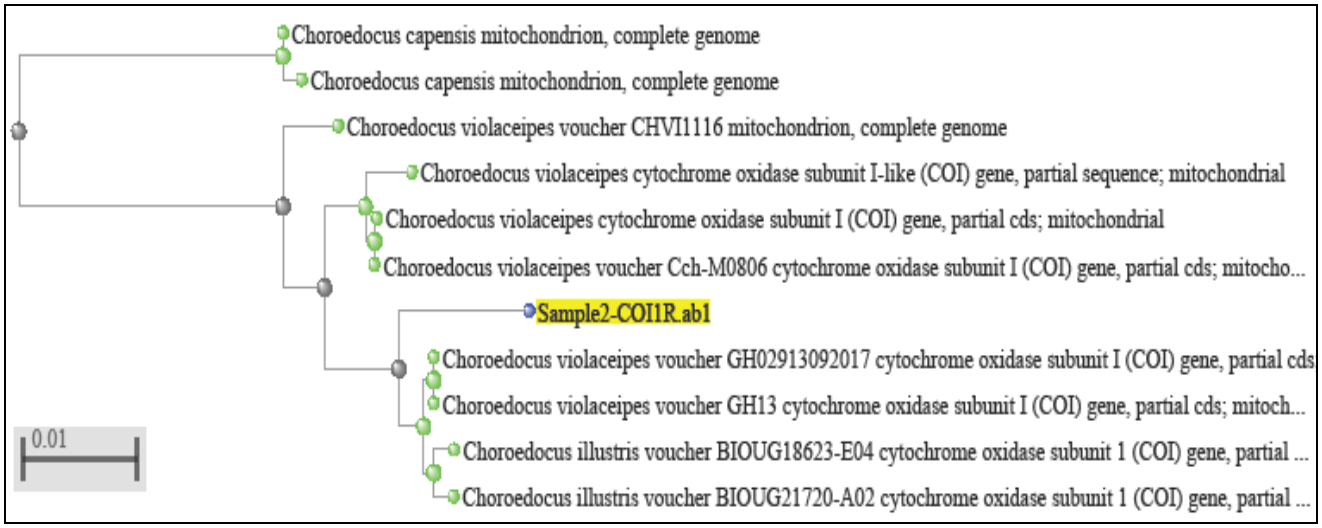


Fig 1: Molecular phylogentic analysis by NJ methods of edible insects of *Choroedocus illustris*

Table 2: BLAST analysis of *Chondracris rosea* with voucher specimens in Gene bank

S. No	Specimen Name	Total Score	Query Cover	E value	Per identification	Accession
1	Chondracris rosea	1067	93 %	0.0	98.62%	MK007252.1
2	Chondracris rosea	1067	99%	0.0	96.90%	GU249619.1

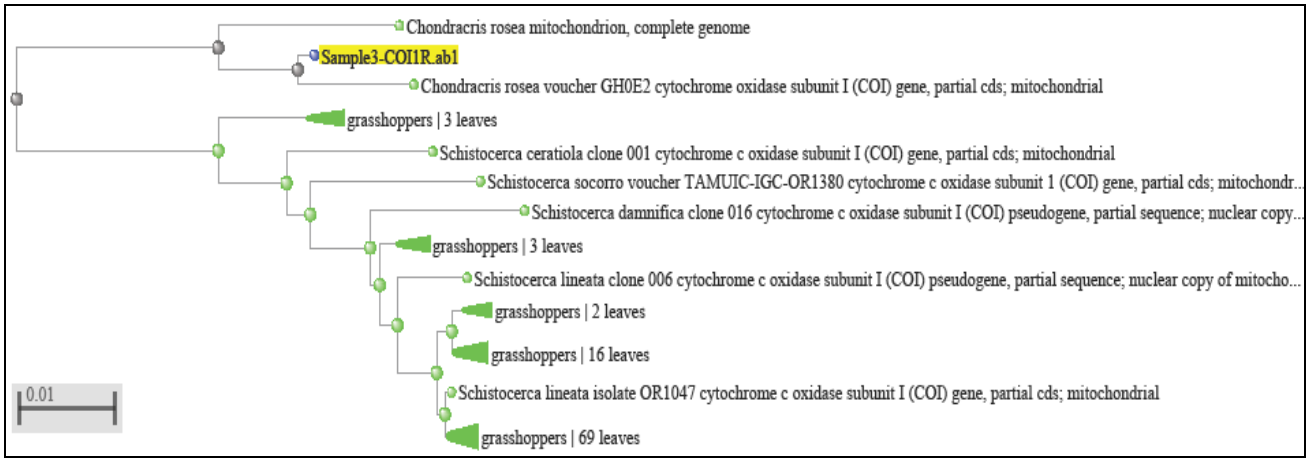


Fig 2: Molecular phylogentic analysis by NN methods of edible insects of *Chondracris rosea*

Table 3: BLAST analysis of *Samia ricini* with voucher specimens in Gene bank

S. No	Specimen Name	Total Score	Query Cover	E value	Per identification	Accession
1	*Samia ricini*	1210	99%	0.0	100%	MN120775.1
2	*Samia ricini*	1213	100	0.0	99.84%	AH015037
3	*Samia ricini*	1213	100	0.0	99.84%	AB015866.1

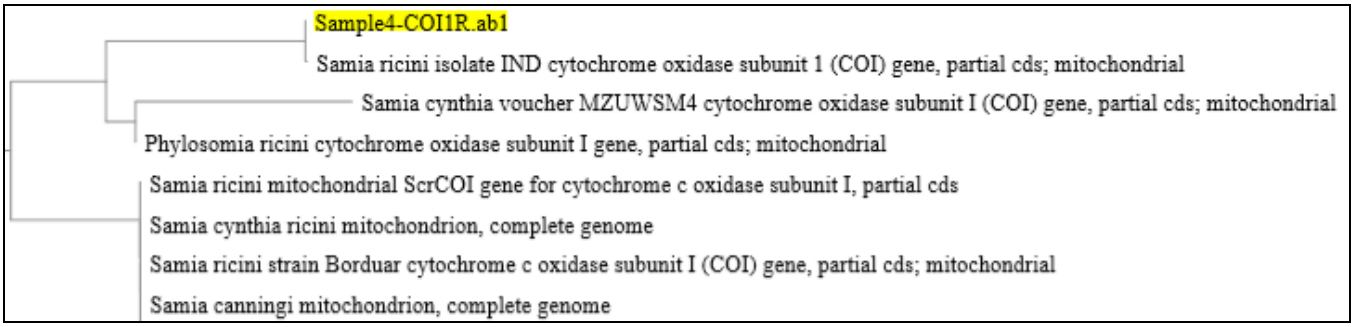


Fig 3: Molecular phylogentic analysis by NN methods of edible insects of *Samia ricini*

Table 4: BLAST analysis of *Tagasta indica* with voucher specimens in Gene bank

S. No	Specimen Name	Total Score	Query Cover	E value	Per identification	Accession
1	Tagasta indica	1092	96%	0.0	95.98%	NC045930.1

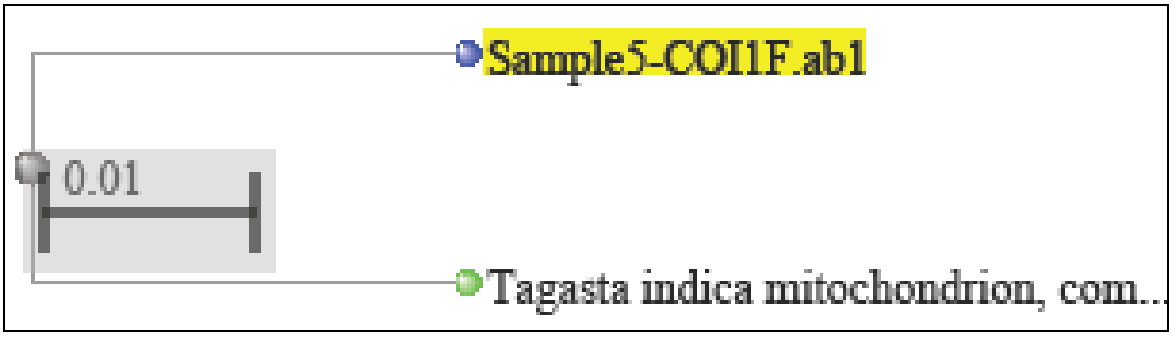


Fig 4: Molecular phylogentic analysis by NJ methods of edible insects of Tagasta indica

Table 5: BLAST analysis of *Odontotermes longignathus* with voucher specimens in Genebank

S. No	Specimen Name	Total Score	Query Cover	E value	Per identification	Accession
1	Odontotermes longignathus	500	77%	3e-137	92.50%	MN205551.1
2	Odontotermes longignathus	500	77%	3e-137	92.50	KY224665.1
3	Odontotermes longignathus	496	72%	4e-136	92.78	KJ934560.1

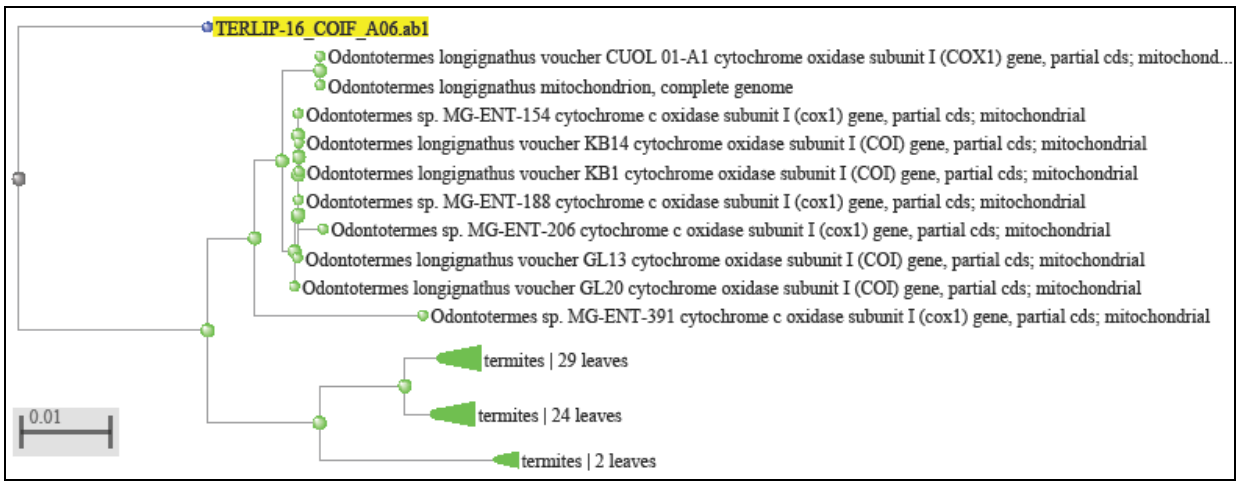


Fig 5: Phylogentic analysis by NJ methods of edible insects of *Odontotermes longignathus*

Table 6: BLAST analysis of *Tessaratoma papillosa* with voucher specimens in Genebank

S. No	Specimen Name	Total Score	Query Cover	E value	Per identification	Accession
1	Tessaratoma papillosa	193	40%	1e-30	80.58	NC037742.1
2	Tessaratoma papillosa	193	40%	1e-30	80.58	AY252948.1

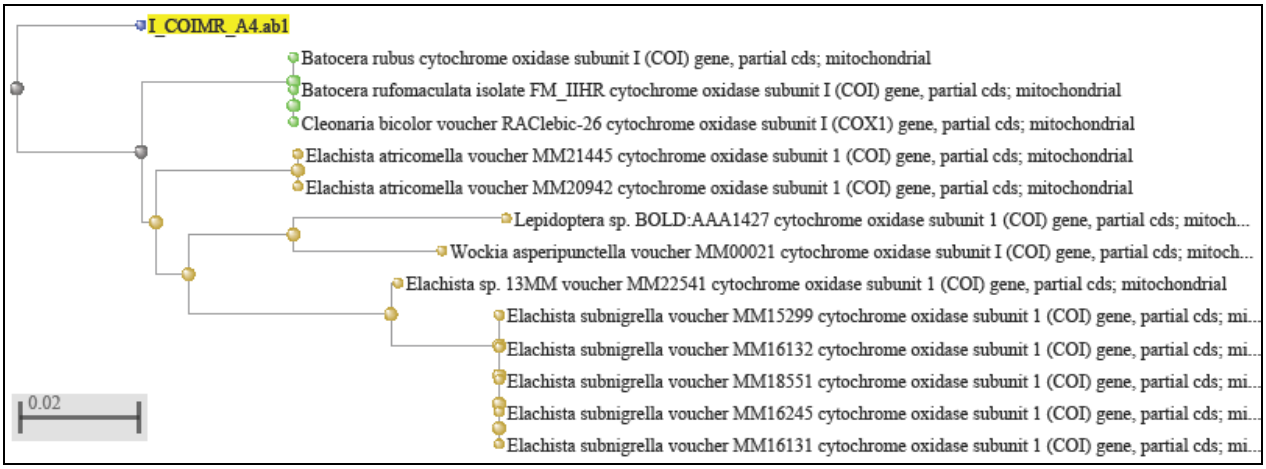


Fig-6: Phylogenic analysis by NJ methods of edible insects of *Batocera rubus*

Table 7: BLAST analysis of *Batocera rubus* with voucher specimens in Gene bank

S. No	Specimen Name	Total Score	Query Cover	E value	Per identification	Accession
1	Batocera rubus	265	59	1e-66	92.47	KT314213.1
2	Batocera rubus	265	59	1e-66	92.47	KX09090.1
3	Batocera rubus	211	58	1e-50	87.57	MK689189.1

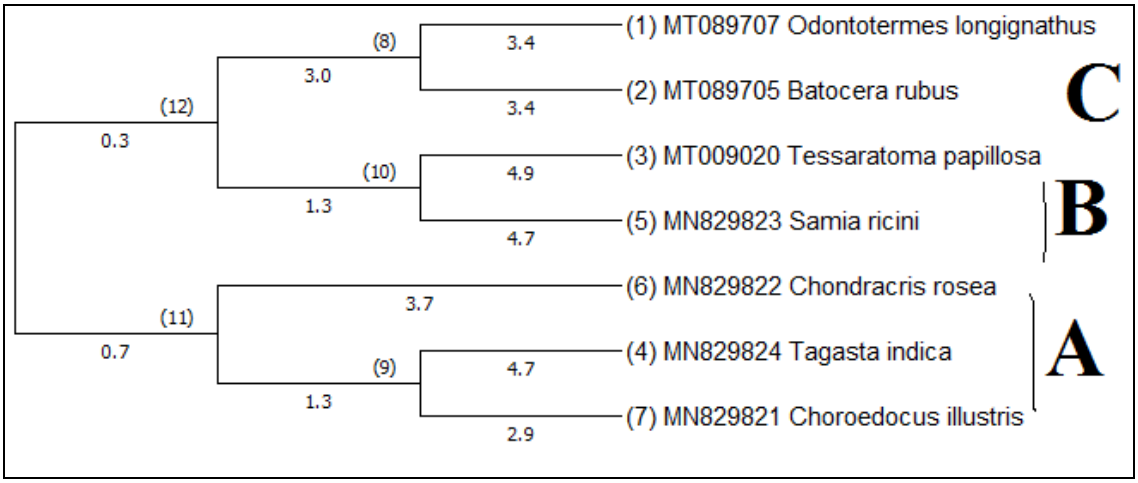


Fig 7: Neighbor joining (NJ) tree of the seven edible insects based on COI gene sequences.

Table 8: List of species name and Gene Bank accession numbers

S.No	Order / Family	Species Name	Gene Bank Accession number
1	Orthropoda/	*Choroedocus illustris* (Grasshopper)	MN829821
2	Acrididae	*Chondracris rosea* (Grasshopper)	MN829822
3	Orthoptera/ pyrgomorphidae	*Tagasta indica* (Short leg Grasshopper)	MN829824
4	Lepidoptera/ Saturniidae	*Samia ricini* (Silk worm)	MN829823
5	Isoptera/ Termitidae	*Odontotermes longignathus* (Wings Termite)	MT009019
6	Hemiptera/ Tessaratomidae	*Tessaratoma papillosa* (Sting bugs)	MT009020
7	Coleoptera/ Cerambycidae	*Batocera rubus* (wood borer)	MT089705

Table 9: Percentage Pair wise distances between edible insects based on COI gene sequences.

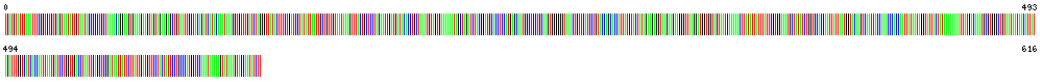
	1	2	3	4	5	6
1.MT089707 Odontotermes longignathus						
2.MT089705 Batocera rubus	6.76					
3.MT009020 Tessaratoma papillosa	13.74	9.69				
4.MN829824 Tagasta indica	14.46	17.72	9.91			
5.MN829823 Samia ricini	14.06	12.22	9.60	12.41		
6.MN829822 Chondracris rosea	8.55	12.47	12.08	10.15	10.43	
7.MN829821 Choroedocus illustris	10.34	9.37	13.67	7.65	10.53	7.47

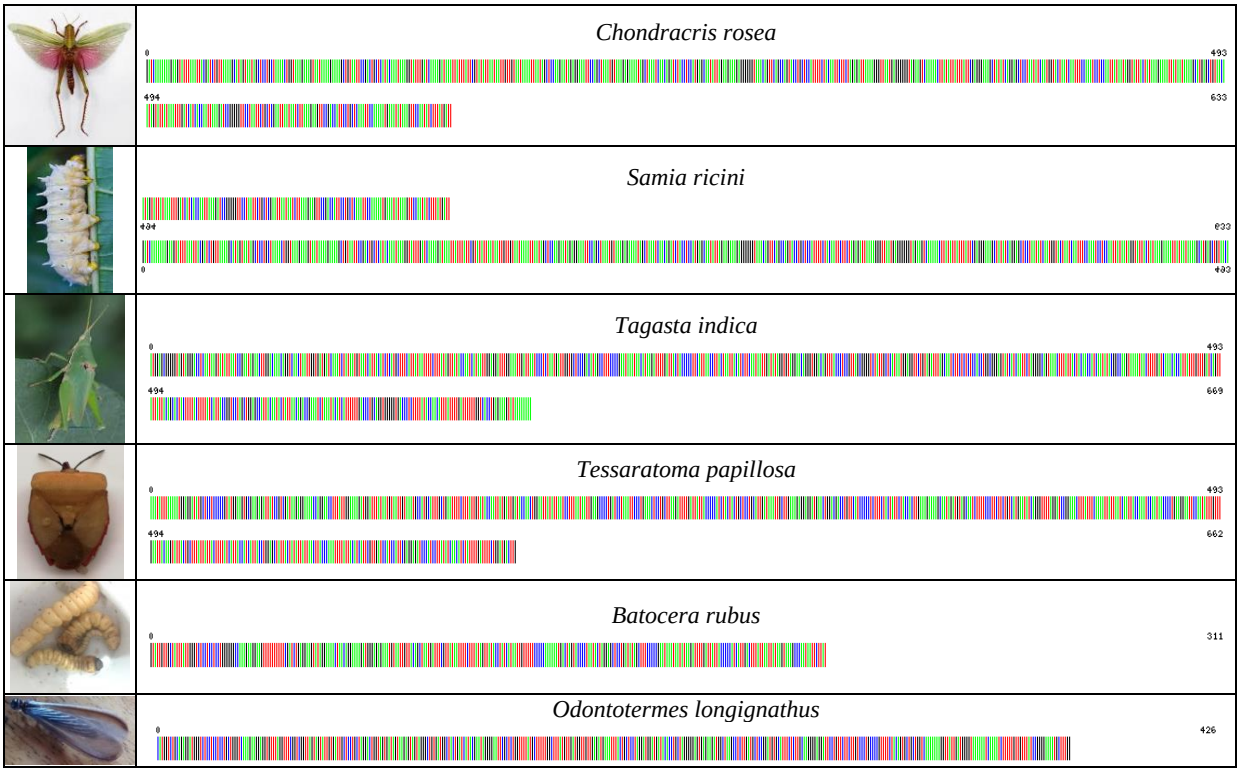
Table 10: Sequence composition, Summary statistics for nucleotide frequency distribution are provided in the table below.

Nucleotides	Min	Mean	Max	SE
G %	16.57	22.92	29.27	4.49
C %	16.16	17.63	19.10	1.04
A %	21.31	26.16	31.04	3.44
T %	33.26	33.27	33.28	0.01
GC %	35.67	40.46	45.43	3.45
GC % Codon pos1	45.29	46.42	47.55	0.799
GC % Codon Pos 2	40.62	41.44	424.25	.057
GC % Codon Pos 3	21.08	33.78	46.48	8.98

Table 11: List of DNA barcode of edible species

Choroedocus illustris





Conclusion

In this study, we have analyzed and documented only 7 edible insects that were collected from Nagaland, but there about 2000 species that have been documented in India. The comprehensive data generated from present study would be useful in further understanding of the biodiversity of edible insects associated with other regions of country and this study would certainly have implication for edible insects for development of diagnostic guide at molecular level.

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